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Mutations in the Fas antigen gene in *lpr* mice

Shigekazu Nagata

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Key words: apoptosis / early transposable element / Fas antigen / lymphoproliferation / T cell development

MAMMALIAN DEVELOPMENT is controlled not only by proliferation and differentiation of cells but also by cell death.[1] There are two death processes, apoptosis and necrosis.[2,3] The death of cells during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy and normal tissue turnover is called programmed cell death. Most of the programmed cell deaths which occur during mammalian development proceed by apoptosis. Apoptosis can be morphologically and biochemically distinguished from necrosis, which occurs during pathological cell death as a result of injury, complement attack, severe hypoxia, hyperthermia, lytic viral infection and exposure to a variety of toxins. Apoptosis is accompanied by condensation and segmentation of nuclei, loss of plasma membrane microvilli and extensive degradation of chromosomal DNA into nucleosome units.

In addition to apoptosis during development (programmed cell death), apoptosis functions in other systems. For example, in the immune system, the death of thymocytes induced through their antigen-receptor complex or by glucocorticoid occurs by an apoptotic process. Sometimes, tumor regression is mediated by apoptosis. Cytotoxic T cells or natural killer cells (NK) as well as tumor necrosis factor (TNF) or lymphotoxin induce apoptosis in the target cells.[4] Furthermore, low doses of U.V. or γ-ray irradiation, or antitumor chemical drugs also causes apoptosis of tumor cells.[2,5]

Programmed cell death has been extensively studied in the live nematode, *Caenorhabditis elegans* (*C. elegans*),[6] in which the division and death of cells can be followed under the microscope. Many mutants in the death process have been identified and molecular analyses of the mutants indicated that many gene products are involved in various aspects of cell death in *C. elegans*. On the other hand, the molecular mechanism of cell death in the mammalian system is poorly understood, despite its importance during development.

Recently, we have shown that the Fas antigen, which is expressed on the cell surface of various mammalian tissues, can mediate apoptosis.[7] Genetic mapping and molecular analysis of the Fas antigene indicated that it is the structural gene for the mouse *lpr* (lymphoproliferation) mutation.[8,9] Here, the molecular properties of the Fas antigen are discussed.

Fas antigen

In 1989, Yonehara *et al*[10] and Trauth *et al*[11] independently established mouse monoclonal antibodies with a cytolytic activity against human cells. These antibodies were designated as anti-Fas or anti-APO-1, and were classified as IgM or IgG_3, respectively. Molecular cloning of the Fas[7] and APO-1 antigen[12] cDNAs has indicated that the anti-Fas and APO-1 antibodies recognize an identical antigen (the Fas antigen) which is a cell surface protein homologous to tumor necrosis factor (TNF) and nerve growth factor (NGF) receptors.[13-16] As shown in Figure 1, the members of this family include two TNF receptors (type I or 55K receptor, and type II or 75K receptor),[17,18] the low affinity NGF receptor,[19] the B cell antigen CD40,[20] the T cell antigen-OX40,[21] CD27,[22] 4-1BB[23] and the CD30 antigen expressed in Hodgkin's lymphoma.[24] The extracellular regions of members in this family are

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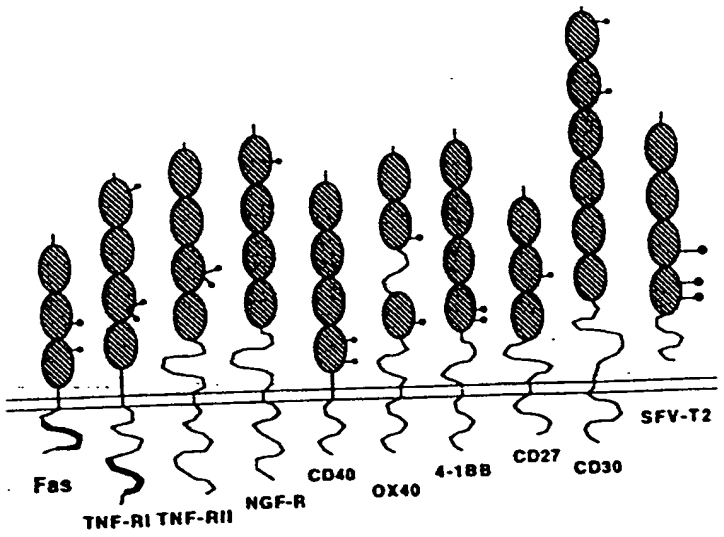

Figure 1. The Fas/TNF/NGF receptor family. Members of the TNF/NGF receptor family are shown schematically. The slashed regions represent cysteine-rich subdomains. Each member of the family contains 3-6 subdomains. The bold areas in the cytoplasmic regions of the Fas antigen and the type I TNF receptor have some similarity. ♀ indicates N-glycosylation site.

rich in cysteine residues, and can be divided into three to six subdomains. Although the amino acid sequence of the extracellular region is relatively conserved (about 24-25% homology) among members, the cytoplasmic region is not conserved except for some homology between the Fas antigen and TNF type 1 receptor (see below). The fact that the Fas antigen is a member of the TNF/NGF receptor family suggests that the Fas antigen is a receptor for an unknown ligand. Recent molecular cloning of the ligand for CD40 (CD40-L)[25,26] indicated that CD40-L is related to TNF,[26,27] suggesting that the Fas-ligand is also a TNF-related molecule. In fact, we have recently isolated the Fas-ligand cDNA. The structural analysis of the cDNA indicated that the Fas-ligand is a TNF-related type II membrane protein.[27a]

To assess the function of the Fas antigen, mouse cell lines constitutively expressing the human Fas antigen were established using the mouse T cell lymphoma WR19L and the fibroblast cell line L929 as host cells.[7] When these transformed cells were treated with anti-human Fas antibody, cells expressing the human Fas antigen, but not the parental mouse cells, died within 5 h. Examination of the dying cells under electron microscope revealed extensive condensation and fragmentation of nuclei which is characteristic of apoptosis. The chromosomal DNA started to degrade in a laddered fashion after a 2 h incubation with the anti-Fas antibody. A human Fas antigen expression plasmid has also been introduced into a mouse IL-3 (interleukin-3)-dependent myeloid leukemia FDC-P1 cell line.[28] Although the transformed cells died by IL-3 depletion, they required 36 h, as observed with the parental FDC-P1 cells. On the other hand, exposure to the anti-human Fas antibody killed the cells within 5 h in the presence of IL-3. From these results, we concluded that the Fas antigen actively mediates the apoptotic signal into cells. Apoptosis was originally defined as a process which requires protein and RNA synthesis.[3] However, there are many examples of apoptosis which is not affected by inhibitors for protein or RNA synthesis. The Fas antigen-mediated apoptosis is a latter example. In L929 transformants, the cytotoxic activity of the anti-Fas antibody was seen only in the presence of actinomycin D, whereas in WR19L or FDC-P1 cell transformants, the antibody alone was sufficient to induce cell death, suggesting that L929 cells express some labile protein(s) which inhibits the Fas antigen-mediated apoptosis.[7,28,29]

Signal transduction mediated by the Fas antigen

The apoptotic signal by the Fas antigen is induced by the binding of anti-Fas antibody or anti-APO-1 antibody to the Fas antigen. The anti-Fas antibody is an IgM class antibody which is an immunoglobulin pentamer, while the anti-APO-1 antibody is an IgG_3 class antibody which tends to aggregate. The $F(ab')_2$ fragment of the anti-APO-1 antibody or other isotypes of the anti-APO-1 antibody hardly induce apoptosis of cells expressing the Fas antigen.[30] On the other hand, the cytotoxic activity of the inactive anti-APO-1 antibody could be reconstituted by cross-linking the antigen with the second antibody or protein A. These results indicate that dimerization of the Fas antigen is not sufficient to activate the Fas antigen to transduce the apoptotic signal. The crosslinking of at least three Fas antigen molecules may be a biologically relevant complex in the generation of an intracellular signal.

The cytoplasmic domain of the Fas antigen consists of 145 amino acids, in which no motif for enzymatic activity such as kinases or phosphatase can be found. However, about 70 amino acids in this region have significant similarity with part of the cytoplasmic region of the type I, but not with the type II TNF receptor. TNF has numerous biological functions, including cytotoxic and proliferative activities.[31] Tartaglia and Goeddel[32] have shown that the type I receptor is responsible for the cytotoxic activity of TNF, while the type II receptor mediates the proliferation signal in thymocytes. The similarity of the Fas antigen and TNF type I receptor in their cytoplasmic regions therefore suggested an important role of this domain for apoptotic signal transduction. In fact, analyses of serial deletion mutants of the Fas antigen from the C-terminus indicated that the domain conserved between the Fas antigen and TNF type I receptor is essential for the function of the Fas antigen.[29] Furthermore, this analysis revealed an inhibitory domain for apoptosis in the C-terminus of the Fas antigen. That is, a Fas antigen mutant lacking 15 amino acids from the C-terminus was an up-mutant, in which about 10-times less of the anti-Fas antibody than that required for the wild-type Fas antigen is sufficient to induce apoptosis. Furthermore, actinomycin D was not required for this mutant to mediate apoptosis in L929 cells.[29] In summary, the cytoplasmic region of the Fas antigen can be divided into two domains, a signal transducing domain of about 70 amino acids in the middle, and a regulatory domain at the C-terminus where the inhibitory protein seems to interact (Figure 2).

Currently, the kinds of signaling molecules involved in Fas antigen-mediated apoptosis are unknown (Figure 2). The growth and differentiation of cells are controlled by signals such as activation of kinases, Ca^{2+} mobilization of cAMP formation, which are stimulated by growth and differentiation factors. The Fas antigen, a putative death factor receptor, may activate a similar signal transducer, or utilize a completely different set of molecules. Since overexpression of the *bcl-2* oncogene product partially inhibits the Fas antigen-mediated apoptosis,[28] *bcl-2* should interact somewhere in the signal-transducing pathway activated by the Fas antigen system.

Expression of the Fas antigen

Activated human T and B cells abundantly express the Fas antigen.[11] Lymphoblastoid cells transformed with human T cell leukemia virus (HTLV)-1,[33] human immunodeficiency virus (HIV)[34] or Epstein-Barr virus (EBV)[35] also highly express functional Fas antigen. Some other tumor cell lines such as human myeloid leukemia U937,[10] human squamous carcinoma CHU-2[7] and SV40-transformed mouse macrophage BAM3 cells[36] express the Fas antigen,

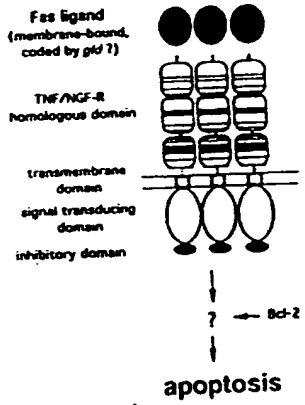

Figure 2. Fas antigen-mediated apoptosis. Putative Fas ligands and the Fas antigen are shown schematically. The extracellular region of the Fas antigen is divided into three cysteine-rich subdomains. The bars in this region indicate the cysteine residues. The cytoplasmic region of the Fas antigen is divided into the signal transducing and inhibitory domains.

although the expression level is low compared with the lymphoblastoid cell lines. The expression of the Fas antigen is upregulated by interferon-γ (IFN-γ)[7,36] which may explain the synergistic effect of the IFN-γ on the cytotoxic activity of the anti-Fas antibody.[10]

The tissue distribution of the Fas antigen was examined in the mouse.[36] Fas antigen mRNA was detected in the thymus, heart, liver and ovary of 8-week-old adult mice, but not in the brain, bone marrow and spleen. In agreement with the expression of Fas antigen mRNA in the thymus, most mouse thymocytes express the Fas antigen on the cell surface.[37]

The Fas antigen gene in *lpr* mice

Southern hybridization of genomic DNA indicated that there is only one chromosomal gene for the Fas antigen in human and mouse chromosomes (unpublished results). *In situ* hybridization localized the human gene on chromosome 10q24.1,[38] and interspecific backcross analysis indicated that the mouse Fas antigen gene is in the region of chromosome 19 which is homologous to the human 10q24.1.[36] Referring the location of the mouse Fas antigen gene to the Genomic Database (GBASE) maintained in the Jackson Laboratory (Bar Harbor, Maine), it was found that the Fas antigen gene is close to the *lpr* locus.[39] We thought that the phenotypes of *lpr* mice may be explained by a mutation in the Fas antigen, since more than 95% of precursor T cells die by apoptosis in the thymus during T cell development.[39] If the Fas antigen plays a role in this process, its mutation may cause the lymphadenopathy and autoimmune disease observed in *lpr* mice.[41]

Two *lpr* mutations, *lpr* and *lpr*^{cg} are known. These mutants have a similar phenotype, but *lpr*^{cg} slightly complements the *gld* mutations in double heterozygotes between *lpr* and *gld* mutations.[42] Northern hybridization of the thymus from *lpr* mice showed little expression of the Fas antigen mRNA.[8] Accordingly, flow cytometry using anti-mouse Fas antibody hardly detected the Fas antigen on the thymocytes from *lpr* mice.[37] Since Southern hybridization of the chromosomal DNA suggested a distinct rearrangement of the Fas antigen gene in *lpr* mice, the chromosomal gene was molecularly cloned from the wild-type and *lpr* mice.[9] The mouse Fas antigen gene consists of over 70 kb, and is split by 9 exons (unpublished results). Restriction enzyme mapping of the Fas antigen gene from *lpr* mice indicated that the promoter and exons of the Fas antigen gene in this mouse are intact. However, the insertion of an early transposable element (ETn) of 5.4 kb was found in intron 2 of the Fas antigen gene (Figure 3). The ETn is a mouse

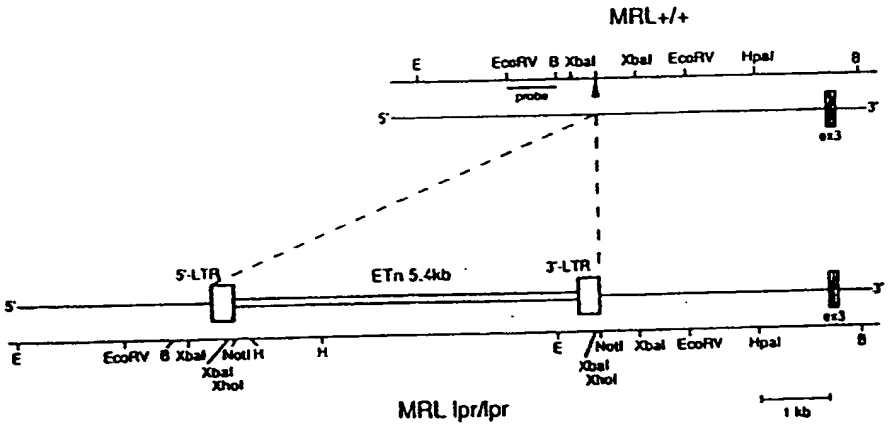

Figure 3. Insertion of an early transposable element in intron 2 of the Fas antigen gene of *lpr* mouse. A restriction map of intron 2 of the Fas antigen genes in wild-type (top) and *lpr* (bottom) mice of the MRL strain. The filled arrowhead in the wild-type gene indicates the point where the ETn is inserted. The shaded boxes represent exon 3, while the open boxes in the *lpr* gene indicate the LTR of the ETn. The recognition sites for major restriction enzymes are indicated using the following abbreviations: E, EcoR1; B, BamHI; H, HindIII.

endogenous retrovirus, of which about 1 000 copies can be found in the mouse genome.[43] Although the ETn does not carry a meaningful open reading frame, it has long terminal repeat (LTR) sequences (about 300 bp) at both the 5' and 3' termini. This LTR sequence contains a poly(A) adenylation signal (AATAAA), and transcription terminates at this region. In fact, short mRNAs of about 1.0 kb coding for exons 1 and 2 of the Fas antigen gene were abundant in the thymus and liver of the *lpr* mice.[9] Furthermore, inserting of the ETn into an intron of a mammalian expression vector dramatically, but not completely, reduced the expression efficiency in mammalian cells. From these results, we concluded that the *lpr* mice have a defect in the expression of the Fas antigen due to the insertion of ETn in the intron 2. However, this is a leaky mutation, and the Fas antigen can be expressed in this mutant at about 1-2% of the wild-type level.

In contrast to the *lpr* mice, *lpr*^{cg} mice express the Fas antigen mRNA of normal size as abundantly as the wild-type.[8] However, this mRNA carries a point mutation of T to A, which causes the replacement of isoleucine with asparagine in the cytoplasmic region of the Fas antigen. This mutation is in the domain which has similarity with the TNF type I receptor mentioned above, and abolished the ability of the Fas antigen to transduce the apoptotic signal.[8] Furthermore, when the corresponding amino acid (valine-238) of the human Fas antigen was mutated to asparagine, it could not transduce the apoptotic signal into cells.[29]

Perspectives

After identification of the Fas antigen as a cell-surface protein which mediates apoptosis, considerable progress has been made regarding the physiological role of the Fas antigen. Our finding that the Fas antigen gene is the structural gene for *lpr* mutation pointed to an important role of the Fas antigen in the development of T cells. However, at which step of the T cell development the Fas antigen is involved remains to be discovered, and this may be a subject for other chapters of this issue. The Fas antigen is expressed in other tissues such as the liver, heart and lung. Although these organs are rather stable, and no apparent phenotypes are seen in these tissues of *lpr* mice, the Fas antigen may also be involved in their development and/or turnover.

The structure of the Fas antigen indicates that it is an orphan receptor for an unknown cytokine. Mice carrying the *gld* mutation show phenotypes similar to the *lpr*, and Allen *et al*[44] have suggested that *gld* and *ipr* are mutations of an interacting pair of molecules. This indicated that *gld* codes for the Fas ligand, and regulates the development of T cells. On the other hand, Rouvier *et al*,[45] have shown that one cytotoxic T cell line expresses the Fas ligand on its surface. These results imply that a molecule (Fas ligand) involved in the T cell development plays an important role in killing tumor cells. Molecular cloning of the Fas ligand would clarify these points.

As growth factor receptors transduce the growth signal, the Fas antigen seems to transduce the death signal into cells. Elucidation of the apoptotic signal transduction mechanism mediated by the Fas antigen may reveal a novel mechanism. The availability of natural mutants of the Fas antigen, *lpr* and *lpr*^{cg} would greatly help elucidate such a mechanism.

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